SUBSTITUTE FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
13028-002001

U.S. APPLICATION NO. (If Known, see 37 CFR 1.5)
09/807096

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP99/07613	11 October 1999	9 October 1998

TITLE OF INVENTION
METHOD FOR OBTAINING ACTIVE BETA-NGF

APPLICANT(S) FOR DO/EO/US
Anke Rattenholl, Elisabeth Schwarz and Adelbert Grossmann

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other documents or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

CERTIFICATE OF MAILING BY EXPRESS MAIL Express Mail Label No EL359011525US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231

April 9, 2001 Samantha Bell Samantha Bell

Date of Deposit Signature Typed Name of Person Signing

JC02 Rec'd PCT/PTO 09 APR 2001

APPLICATION NO. (IF KNOWN) 09/807096	INTERNATIONAL APPLICATION NO. PCT/EP99/07613	ATTORNEY'S DOCKET NUMBER 13028-002001

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)- (5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... **$860**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710**	
nternational preliminary examination fee paid to USPTO (37 CFR 1.482) out all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690**	
nternational preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00
Surcharge of **$130** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$0.00

Claims	Number Filed	Number Extra	Rate	
otal Claims	19 - 20 =	0	x **$18**	$0.00
ndependent Claims	3 - 3 =	0	x **$80**	$0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ **$270**	$0.00
TOTAL OF ABOVE CALCULATIONS =				$860.00
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are educed by 1/2.				$0.00
SUBTOTAL =				$430.00
rocessing fee of **$130** for furnishing the English Translation later than ☐ 20 ☐ 30 onths from the earliest claimed priority date (37 CFR 1.492(f))				$0.00
TOTAL NATIONAL FEE =				$430.00
e for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be companied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$0.00
TOTAL FEES ENCLOSED =				$430.00
			Amount to be refunded:	$
			Charged:	$

☒ A check in the amount of $430.00 to cover the above fees is enclosed.
☐ Please charge my Deposit Account No. 06-1050 in the amount of $0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.

TE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

ND ALL CORRESPONDENCE TO:

Rocky Tsao
H & RICHARDSON P.C.
Franklin Street
ton, MA 02110-2804
7) 542-5070 *phone*
7) 542-8906 *facsimile*

SIGNATURE: Y. Rocky Tsao

NAME: Y. Rocky Tsao

REGISTRATION NUMBER: 34,053

0178036.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Anke Rattenholl et al. Art Unit : Unknown
Serial No. : Unassigned Examiner : Unknown
Filed : Herewith
Title : METHOD FOR OBTAINING ACTIVE BETA-NGF

Box PCT
Commissioner for Patents
Washington, D.C. 20231

VERIFIED STATEMENT UNDER 37 CFR §1.821(f)

I, Jennifer H. Payne, declare that I personally prepared the paper and the computer-readable copy of the Sequence Listing filed herewith for the above-identified application and that the content of both is the same.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of The United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: April 8, 2001

Jennifer H. Payne

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
(617) 542-5070 telephone
(617) 542-8906 facsimile

20221093.doc

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April 9, 2001
Date of Deposit

Samantha Bell
Signature

Samantha Bell
Typed or Printed Name of Person Signing Certificate

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Anke Rattenholl et al.	Art Unit	: Unknown
Serial No.	: Unassigned	Examiner	: Unknown
Filed	: Herewith		
Title	: METHOD FOR OBTAINING ACTIVE BETA-NGF		

Box PCT
Commissioner for Patents
Washington, D.C. 20231

STATEMENT UNDER 37 CFR §1.821(f)

I hereby state, as required by 37 C.F.R. §1.821(f), that the content of the paper and computer-readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. §§1.821(c) and (e), respectively, are the same.

Respectfully submitted,

Date: 4-9-01

Y. Rocky Tsao
Reg. No. 34,053

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

20221108.doc

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April 9, 2001
Date of Deposit

Samantha Bell
Signature

Samantha Bell
Typed or Printed Name of Person Signing Certificate

SEQUENCE LISTING

<110> Rattenholl, Anke
Schwarz, Elisabeth
Grossmann, Adelbert

<120> METHOD FOR OBTAINING ACTIVE BETA-NGF

<130> 13028-002001

<150> PCT/EP99/07613
<151> 1999-10-11

<150> EP 98119077.0
<151> 1998-10-09

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Gln Thr Arg Asn Ile Thr Val Asp Pro Arg Leu Phe Lys Lys Arg Arg
    50                  55                  60

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Leu Arg Ser Pro Arg Val Leu Phe Ser Thr Gln Pro Pro Arg Glu Ala
65                  70                  75                  80

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Ala Asp Thr Gln Asp Leu Asp Phe Glu Val Gly Gly Ala Ala Pro Phe
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Asn Arg Thr His Arg Ser Lys Arg Ser Ser Ser His Pro Ile Phe His
            100                 105                 110

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Arg Gly Glu Phe Ser Val Cys Asp Ser Val Ser Val Trp Val Gly Asp
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Lys Thr Thr Ala Thr Asp Ile Lys Gly Lys Glu Val Met Val Leu Gly
    130                 135                 140

gag gtg aac att aac aac agt gta ttc aaa cag tac ttt ttt gag acc      480
Glu Val Asn Ile Asn Asn Ser Val Phe Lys Gln Tyr Phe Phe Glu Thr
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Lys Cys Arg Asp Pro Asn Ser Val Asp Ser Gly Cys Arg Gly Ile Asp
                165                 170                 175

tca aag cac tgg aac tca tat tgt acc acg act cac acc ttt gtc aag      576
Ser Lys His Trp Asn Ser Tyr Cys Thr Thr Thr His Thr Phe Val Lys
            180                 185                 190

gcg ctg acc atg gat ggc aag cag gct gcc tgg cgg ttt atc cgg ata      624
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Asn Arg Thr His Arg Ser Lys Arg Ser Ser Ser His Pro Ile Phe His
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115 120 125
Lys Thr Thr Ala Thr Asp Ile Lys Gly Lys Glu Val Met Val Leu Gly
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Glu Val Asn Ile Asn Asn Ser Val Phe Lys Gln Tyr Phe Phe Glu Thr
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#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Anke Rattenholl et al.
Serial No. : 09/807,096
Filed : April 9, 2001
Title : METHOD FOR OBTAINING ACTIVE BETA-NGF

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

VERIFIED STATEMENT UNDER 37 CFR § 1.821(f)

I, Jennifer H. Payne, declare that I personally prepared the paper and the computer-readable copy of the Sequence Listing filed herewith for the above-identified application and that the content of both is the same.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of The United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: November 19, 2001

Jennifer H. Payne

Fish & Richardson P.C.
225 Franklin Street
Boston, Massachusetts 02110-2804
(617) 542-5070 telephone
(617) 542-8906 facsimile

20317716.doc

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Express Mail Label No. EL245447978US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, 2900 Crystal Drive, Arlington, VA 22202-3513.

November 19, 2001
Date of Deposit

Signature

Leroy Jenkins
Typed or Printed Name of Person Signing Certificate

Attorney's Docket No.: 13028-002001 / P12999

FIGPCT Rec'd 19 NOV 2001

#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Anke Rattenholl et al.
Serial No. : 09/807,096
Filed : April 9, 2001
Title : METHOD FOR OBTAINING ACTIVE BETA-NGF

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

RESPONSE TO NOTIFICATION TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE AND/OR AMINO ACID SEQUENCES

In response to the communication dated July 30, 2001 (copy enclosed), applicants submit herewith a Sequence Listing in computer readable form as required by 37 CFR § 1.824. In addition, applicants submit a substitute Sequence Listing as required under 37 CFR § 1.823(a) and a statement under 37 CFR §1.821(f).

Applicants respectfully request entry of the paper copy and computer readable copy of the Sequence Listing filed herewith for the instant application. Furthermore, applicants request entry of the following amendments.

In the specification:

Replace the original Sequence Listing with the substitute Sequence Listing filed herewith.

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL245447978US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, 2900 Crystal Drive, Arlington, VA 22202-3513.

November 19, 2001
Date of Deposit

Signature

Leroy Jenkins
Typed or Printed Name of Person Signing Certificate

REMARKS

Applicants hereby submit that the enclosures fulfill the requirements under 37 C.F.R. §1.821-1.825. The amendments in the specification merely replace the original Sequence Listing with a substitute Sequence Listing. No new matter has been added.

Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 11-19-01

Y. Rocky Tsao
Y. Rocky Tsao
Reg. No. 34,053

Fish & Richardson P.C.
225 Franklin Street
Boston, Massachusetts 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

20323828.doc

#3

SEQUENCE LISTING

<110> Rattenholl, Anke
Schwarz, Elisabeth
Grossmann, Adelbert

<120> METHOD FOR OBTAINING ACTIVE BETA-NGF

<130> 13028-002001

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 1               5                  10                  15

caa gtc cac tgg act aaa ctt cag cat tcc ctt gac act gcc ctt cgc        96
Gln Val His Trp Thr Lys Leu Gln His Ser Leu Asp Thr Ala Leu Arg
            20                  25                  30

aga gcc cgc agc gcc ccg gca gcg gcg ata gct gca cgc gtg gcg ggg       144
Arg Ala Arg Ser Ala Pro Ala Ala Ala Ile Ala Ala Arg Val Ala Gly
        35                  40                  45

cag acc cgc aac att act gtg gac ccc agg ctg ttt aaa aag cgg cga       192
Gln Thr Arg Asn Ile Thr Val Asp Pro Arg Leu Phe Lys Lys Arg Arg
    50                  55                  60

ctc cgt tca ccc cgt gtg ctg ttt agc acc cag cct ccc cgt gaa gct       240
Leu Arg Ser Pro Arg Val Leu Phe Ser Thr Gln Pro Pro Arg Glu Ala
65                  70                  75                  80

gca gac act cag gat ctg gac ttc gag gtc ggt ggt gct gcc ccc ttc       288
Ala Asp Thr Gln Asp Leu Asp Phe Glu Val Gly Gly Ala Ala Pro Phe
                85                  90                  95

aac agg act cac agg agc aag cgc tca tca tcc cat ccc atc ttc cac       336
Asn Arg Thr His Arg Ser Lys Arg Ser Ser Ser His Pro Ile Phe His
            100                 105                 110

agg ggc gaa ttc tcg gtg tgt gac agt gtc agc gtg tgg gtt ggg gat       384
Arg Gly Glu Phe Ser Val Cys Asp Ser Val Ser Val Trp Val Gly Asp
        115                 120                 125

aag acc acc gcc aca gat atc aag ggc aag gag gtg atg gtg ttg gga       432
Lys Thr Thr Ala Thr Asp Ile Lys Gly Lys Glu Val Met Val Leu Gly
    130                 135                 140

gag gtg aac att aac aac agt gta ttc aaa cag tac ttt ttt gag acc       480
Glu Val Asn Ile Asn Asn Ser Val Phe Lys Gln Tyr Phe Phe Glu Thr
145                 150                 155                 160

aag tgc cgg gac cca aat tcc gtc gac agc ggg tgc cgg ggc att gac       528
Lys Cys Arg Asp Pro Asn Ser Val Asp Ser Gly Cys Arg Gly Ile Asp
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tca aag cac tgg aac tca tat tgt acc acg act cac acc ttt gtc aag       576
Ser Lys His Trp Asn Ser Tyr Cys Thr Thr Thr His Thr Phe Val Lys
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gcg ctg acc atg gat ggc aag cag gct gcc tgg cgg ttt atc cgg ata       624
Ala Leu Thr Met Asp Gly Lys Gln Ala Ala Trp Arg Phe Ile Arg Ile
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Asp Thr Ala Cys Val Cys Val Leu Ser Arg Lys Ala Val Arg
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PATENT APPLICATION: US/09/807,096 TIME: 13:15:19

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Leu Arg Ser Pro Arg Val Leu Phe Ser Thr Gln Pro Pro Arg Glu Ala
 65                  70                  75                  80
gca gac act cag gat ctg gac ttc gag gtc ggt ggt gct gcc ccc ttc      288
Ala Asp Thr Gln Asp Leu Asp Phe Glu Val Gly Gly Ala Ala Pro Phe
                 85                  90                  95
aac agg act cac agg agc aag cgc tca tca tcc cat ccc atc ttc cac      336
Asn Arg Thr His Arg Ser Lys Arg Ser Ser Ser His Pro Ile Phe His
            100                 105                 110
agg ggc gaa ttc tcg gtg tgt gac agt gtc agc gtg tgg gtt ggg gat      384
Arg Gly Glu Phe Ser Val Cys Asp Ser Val Ser Val Trp Val Gly Asp
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aag acc acc gcc aca gat atc aag ggc aag gag gtg atg gtg ttg gga      432
Lys Thr Thr Ala Thr Asp Ile Lys Gly Lys Glu Val Met Val Leu Gly
    130                 135                 140
gag gtg aac att aac aac agt gta ttc aaa cag tac ttt ttt gag acc      480
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145                 150                 155                 160
aag tgc cgg gac cca aat tcc gtc gac agc ggg tgc cgg ggc att gac      528
Lys Cys Arg Asp Pro Asn Ser Val Asp Ser Gly Cys Arg Gly Ile Asp
                165                 170                 175
tca aag cac tgg aac tca tat tgt acc acg act cac acc ttt gtc aag      576
Ser Lys His Trp Asn Ser Tyr Cys Thr Thr Thr His Thr Phe Val Lys
            180                 185                 190
gcg ctg acc atg gat ggc aag cag gct gcc tgg cgg ttt atc cgg ata      624
Ala Leu Thr Met Asp Gly Lys Gln Ala Ala Trp Arg Phe Ile Arg Ile
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PATENT APPLICATION: US/09/807,096 TIME: 13:15:19

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PCT09

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DATE: 12/21/2001
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<110> APPLICANT: Rattenholl, Anke
Schwarz, Elisabeth
Grossmann, Adelbert

<120> TITLE OF INVENTION: METHOD FOR OBTAINING ACTIVE BETA-NGF

<130> FILE REFERENCE: 13028-002001

<140> CURRENT APPLICATION NUMBER: 09/807,096

<141> CURRENT FILING DATE: 2001-04-09

<150> PRIOR APPLICATION NUMBER: PCT/EP99/07613

<151> PRIOR FILING DATE: 1999-10-11

<150> PRIOR APPLICATION NUMBER: EP 98119077.0

<151> PRIOR FILING DATE: 1998-10-09

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SEQUENCE LISTING

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<120> Method for otaining active beta-NGF

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<151> 1998-10-09

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Anke Rattenholl et al. Art Unit : Unknown
Serial No. : Unassigned Examiner : Unknown
Filed : Herewith
Title : METHOD FOR OBTAINING ACTIVE BETA-NGF

Box PCT
Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the specification:

Replace the original Sequence Listing with the amended substitute Sequence Listing filed herewith. Insert the substitute Sequence Listing following the Drawings of the instant application.

Replace the paragraph beginning at page 14, line 4 with the following rewritten paragraph:

--The following primers were used in the PCR:

Forward primer "FwProNGF":

5'-CG GAA TTC CA|T ATG GAA CCA CAC TCA GAG AGC-3' **(SEQ ID NO: 1)**
Met Glu Pro His Ser Glu Ser **(SEQ ID NO:5)**

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL259011525US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner of Patents, Washington, D.C. 20231.

April 9, 2001
Date of Deposit

Samantha Bell
Signature

Samantha Bell
Typed or Printed Name of Person Signing Certificate

Reverse primer "RevNGF":

5'-CC G|GA TCC TTA TCA TCT CAC AGC CTT TCT AGA-3' **(SEQ ID NO: 2)**

stop stop Arg Val Ala Lys Arg Ser **(SEQ ID NO:6)**

After cloning into the vector, the nucleotide sequence was verified by means of DNA sequencing.--

Replace the paragraph beginning at page 20, line 18 with the following rewritten paragraph:

--To determine the concentration of rh proNGF in the purified samples, an UV spectrum from 240 to 340 nm was taken of the samples dialyzed against 50 mM Na-phosphate pH 7.0, 1 mM EDTA (Fig. 5; the spectrum was obtained using a Beckman DU 640 spectrophotometer). The rh proNGF concentration in the sample was determined by means of absorption at 280 nm. The evaluation was based on a theoretical molar extinction coefficient of 25,680 l/(mol x cm) (calculated according to Gill, S. C., et al., Anal. Biochem. 182 (1989) 319) and a molecular weight of 24,869 Da per monomer (calculated by means of the ExPASy program "pI/Mw" and corrected for three disulfide bonds). The values obtained using the spectrum were in close correlation to the concentrations determined by means of the Bradford method. Molecular weight determination was done using electron spray mass spectrometry. The theoretical mass of recombinant proNGF is 24,869 Da. Experimentally determined were 24,871 Da.--

Replace the paragraph beginning at page 21, line 26 with the following rewritten paragraph:

--3.1 µg of rh proNGF (15 µl rh proNGF in a concentration of 0.21 mg/ml) were loaded onto a Poros 10 R1 column (100 mm x 4 mm; Perseptive Biosystems) equilibrated with 0.13% TFA. The protein was eluted at a flow rate of 0.8 ml/min with a non-linear gradient (0-4 min: 6% B; 4-9 min: 6-30% B; 9-24 min: 30-69% B; 24-25 min: 69-100%

B; 25-30 min: 100% B)) in a period of 33 minutes. As the eluent B there was used 0.1% (v/v) TFA in 80% (v/v) acetonitrile. The absorption at 220 nm was used for detection (Beckman "Gold" HPLC system with analysis software "Gold V 8.10"). Native rh proNGF eluted in a single peak at a retention time of 14.28 min (Fig. 7).--

Replace the paragraph beginning at page 22, line 7 with the following rewritten paragraph:

--For N terminal sequence analysis the solubilized IBs were used which had been roughly purified by means of RP HPLC. The N terminal sequence was determined using an Applied Biosystems 476A protein sequencing device. The following amino acid sequence was obtained:

H_2N-Met-Glu-Pro-His-Ser-Glu-Ser-Asn-Val (SEQ ID NO:7).--

In the claims:

Amend claims 4, 5, and 7 as follows:

--4. (Amended) A method according to claim 1 wherein the naturation is carried out in the presence of a thiol component in its reduced and oxidized form.

5. (Amended) A method according to claim 1 wherein the cleaving off of the prosequence is carried out by means of a protease with a substrate specificity for cleaving after the amino acid arginine.

7. (Amended) A method according to claim 1 wherein guanidinium hydrochloride or urea is used as the denaturing agent.--

Please add claims 10-19:

--10. A method according to claim 2 wherein the naturation is carried out in the presence of a thiol component in its reduced and oxidized form.

11. A method according to claim 3 wherein the naturation is carried out in the presence of a thiol component in its reduced and oxidized form.

12. A method according to claim 2 wherein the cleaving off of the prosequence is carried out by means of a protease with a substrate specificity for cleaving after the amino acid arginine.

13. A method according to claim 3 wherein the cleaving off of the prosequence is carried out by means of a protease with a substrate specificity for cleaving after the amino acid arginine.

14. A method according to claim 4 wherein the cleaving off of the prosequence is carried out by means of a protease with a substrate specificity for cleaving after the amino acid arginine.

15. A method according to claim 2 wherein guanidinium hydrochloride or urea is used as the denaturing agent.

16. A method according to claim 3 wherein guanidinium hydrochloride or urea is used as the denaturing agent.

17. A method according to claim 4 wherein guanidinium hydrochloride or urea is used as the denaturing agent.

18. A method according to claim 5 wherein guanidinium hydrochloride or urea is used as the denaturing agent.

19. A method according to claim 6 wherein guanidinium hydrochloride or urea is used as the denaturing agent.--

REMARKS

The amendments to the specification have been made to insert sequence identifiers, to amend typographical errors, and to replace the original Sequence Listing with an amended substitute Sequence Listing. In particular, the amended substitute Sequence Listing includes the amino acid sequences shown on pages 14 and 22 of the translated specification.

All amendments to the claims have been made to remove multiple dependency while conserving the claimed subject matter. More specifically, new claims 10-19, are subsets of original claims 4, 5, and 7. Thus, no new matter has been introduced.

Attached is a marked-up version of the changes being made by the current amendment.

Claims 1-19 are now pending. Applicant submits that all of the claims are now in condition for examination, which action is requested. Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 4-9-01

Y. Rocky Tsao
Reg. No. 34,053

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

Version with markings to show changes made

In the specification:

Paragraph beginning at page 14, line 4 has been amended as follows:

The following primers were used in the PCR:

Forward primer "FwProNGF":

5'-CG GAA TTC CA|T ATG GAA CCA CAC TCA GAG AGC-3' **(SEQ ID NO: 1)**

Met Glu Pro His Ser Glu Ser **(SEQ ID NO:5)**

Reverse primer "RevNGF":

5'-CC G|GA TCC TTA TCA TCT CAC AGC CTT TCT AGA-3' **(SEQ ID NO: 2)**

stop stop Arg Val Ala Lys Arg Ser **(SEQ ID NO:6)**

After cloning into the vector, the nucleotide sequence was verified by means of DNA sequencing.

Paragraph beginning at page 20, line 18 has been amended as follows:

To determine the concentration of rh proNGF in the purified samples, an UV spectrum from 240 to 340 nm was taken of the samples dialyzed against 50 mM Na-phosphate pH 7.0, 1 mM EDTA (Fig. 5; the spectrum was obtained using a Beckman DU 640 spectrophotometer). The rh proNGF concentration in the sample was determined by means of absorption at 280 nm. The evaluation was based on a theoretical molar extinction coefficient of 25,680 l/(mol x cm) (calculated according to Gill, S. C., et al., Anal. Biochem. 182 (1989) 319) and a molecular weight of 24,869 Da per monomer (calculated by means of the ExPASy program "pI/Mw" and corrected for three disulfide bonds). The values obtained using the spectrum were in close correlation to the

concentrations determined by means of the Bradford method. Molecular weight determination was done using electron spray mass spectrometry. The theoretical mass of recombinant proNGF is [24,689] 24,869 Da. Experimentally determined were 24,871 Da.

Paragraph beginning at page 21, line 26 has been amended as follows:

3.1 µg of rh [proMGF] proNGF (15 µl rh proNGF in a concentration of 0.21 mg/ml) were loaded onto a Poros 10 R1 column (100 mm x 4 mm; Perseptive Biosystems) equilibrated with 0.13% TFA. The protein was eluted at a flow rate of 0.8 ml/min with a non-linear gradient (0-4 min: 6% B; 4-9 min: 6-30% B; 9-24 min: 30-69% B; 24-25 min: 69-100% B; 25-30 min: 100% B)) in a period of 33 minutes. As the eluent B there was used 0.1% (v/v) TFA in 80% (v/v) acetonitrile. The absorption at 220 nm was used for detection (Beckman "Gold" HPLC system with analysis software "Gold V 8.10"). Native rh proNGF eluted in a single peak at a retention time of 14.28 min (Fig. 7).

Paragraph beginning at page 22, line 7 has been amended as follows:

For N terminal sequence analysis the solubilized IBs were used which had been roughly purified by means of RP HPLC. The N terminal sequence was determined using an Applied Biosystems 476A protein sequencing device. The following amino acid sequence was obtained:

H_2N-Met-Glu-Pro-His-Ser-Glu-Ser-Asn-Val (SEQ ID NO:7).

In the claims:

Claims 4, 5, and 7 have been amended as follows:

4. (Amended) A method according to [any of the claims 1 to 3] claim 1 wherein the naturation is carried out in the presence of a thiol component in its reduced and oxidized form.

5. (Amended) A method according to [any of the claims 1 to 4] claim 1 wherein the cleaving off of the prosequence is carried out by means of a protease with a substrate specificity for cleaving after the amino acid arginine.

7. (Amended) A method according to [any of the claims 1 to 6] claim 1 wherein guanidinium hydrochloride or urea is used as the denaturing agent.

PCT/EP99/07613
Applicant: RUDOLPH, Rainer et al.

Method for obtaining active ß-NGF

The present invention relates to a method for the preparation of ß-NGF by naturation of denatured inactive proNGF and cleavage of the pro sequence.

Nerve growth factor (ß-NGF) is a neurotrophic factor required for the growth and survival of sympathetic and sensory neurons (Levi-Montalcini, R., Science 237 (1987) 1154; Thoenen, H., et al., Physiol. Rev. 60 (1980) 1284; Yankner, B. A., et al., Annu. Rev. Biochem. 51 (1982) 845). Furthermore, ß-NGF promotes the growth, differentiation and vitality of cholinergic neurons of the central nervous system (Hefti, F. J., J. Neurobiol. 25 (1994) 1418). Possible therapeutic indications for recombinant human nerve growth factor include peripheral sensory neuropathies, e.g. associated with diabetes or as a possible side effect in AIDS therapy. Other indications for rh ß-NGF are central neuropathies, e.g. Alzheimer's disease. In this case, the loss of memory is the result of a loss of cholinergic neurons.

Human ß-NGF is translated as a preproprotein consisting of 241 amino acids. The prepeptide (18 amino acids) is cleaved off during translocation into the endoplasmic reticulum (ER), while the resulting proprotein is subsequently processed at its N an C terminus (removal of the prosequence (103 amino acids) and the last two amino acids). Therefore, mature human NGF contains 118 amino acids. It shows homology to murine ß-NGF and differs from this protein only by 12 amino acid

exchanges. For conducting clinical studies or a possible use as therapeutic, the ß-NGFs must be available in high amounts. A natural source of higher amounts of this factor are the submaxillary glands of mice. These preparations, however, are heterogeneous mixtures of different dimers and are unsuitable for therapeutic use. Furthermore, it is desirable to administer the human form of the protein to the patients. In human tissue, however, neurotrophic factors are present only in minute concentrations.

Therefore, to use ß-NGF as a therapeutic agent the preparation of the protein by means of recombination is the only possibility. This may be achieved in two ways: by a recombinant expression either in cell cultures or in bacteria. Eukaryotic cell expression systems tend to provide only very low amount of proteins and are relatively expensive (Barnett, J., et al., J. Neurochem. 57 (1991) 1052; Schmelzer, C. H., et al., J. Neurochem. 59 (1992) 1675; US 5,683,894).

In contrast, prokaryotic expression systems provide high amounts of the desired protein. However, in contrast to eukaryotic expression systems bacteria are unable to process the precursor proteins in the correct manner. As in the expression of many other recombinant mammalian genes, the production of recombinant ß-NGFs in bacteria results in a biologically inactive translation product which is then accumulated in the cell in the form of aggregates (so-called inclusion bodies (IBs)).

Naturation of mature ß-NGF from such inclusion bodies, however, is only possible in the case of very low protein concentrations (below 10 µg/ml) and very low yields (up to

about 10%). Such methods are for example described in EP-A 0 544 293, US patent 5,606,031, US patent 5,235,043, as well as WO 97/47735. The naturation via sulfitolysis of neurotrophic factors of the NGF/BDNF family is described in WO 95/30686.

In WO 97/47735 there is described an improved method for the naturation of proteins. In this method, the inactive protein is dissolved in a solution of a denaturing agent having a denaturing concentration in the presence of a low molecular weight substance which contains thiol groups. Afterwards, the dissolved protein is transferred from the strongly denaturing solution into another solution which is not or only weakly denaturing in which it assumes a biologically active conformation wherein the disulfide bonds are opened by means of the thiol component and subsequently are formed newly in the protein in a manner that the protein assumes a conformation which has biological activity. Using an improved method of this type, a yield of naturation of ß-NGF of about 10% may be achieved.

It is an object of the present invention to provide an improved method for the preparation of ß-NGF which is simple and provides active NGF in a high yield.

This object has been solved by providing a method for the preparation of a biologically active ß-NGF by means of naturation of the pro form present in its inactive form and having a very poor solubility wherein the pro form preferably is available in the form of inclusion bodies after recombinant preparation in prokaryotes, said method being characterized by dissolving proNGF in its inactive form which has a poor solubility in a solution of a denaturing agent in a denaturing concentration , transferring proNGF into a

solution which is not or weakly denaturing maintaining the solubility wherein the dissolved denatured proNGF assumes a biologically active conformation which is determined by the disulfide bonds present in the native NGF, and afterwards removing the prosequence whereby active NGF is obtained which may be isolated.

Surprisingly, it has been shown that during naturation of inactive ß-NGF in vitro the prosequence has an essential and positive effect on the naturation process and according to the present invention it is possible to perform the renaturation in the most simple manner and thereby achieve yields of natured active ß-NGF which have not been known so far and have not been deemed possible.

The term "proNGF" means ß-NGF which is linked to its prosequence at its N terminus. According to the present invention, there may be used as said prosequence either the whole prosequence (US patent 5,683,894; Ullrich, A., et al., Nature 303 (1983) 821; SWISS-PROT protein sequence database No. P01138) or portions thereof, preferably complete domains. Suter et al. (EMBO J. 10, 2395 (1991)) have performed a detailed study of the in vivo function of the propeptide of murine ß-NGF on the basis of correct secretion in a COS-7 cell culture system. For this purpose, the prosequence has been divided into five regions. Mutants have been prepared having deletions in one or more of these sequences. It has been found that the sequence regions containing amino acids -52 to -26 ("domain I") as well as -6 to -1 ("domain II") are essential for the expression and secretion of biologically active ß-NGF. Domain I is essential for the expression while domain II is required for correct proteolytic processing. Surprisingly, it has been shown that proNGF has an activity

in vivo analogous to ß-NGF. Therefore, proNGF may also be used as a therapeutic.

Inactive proNGF showing a poor solubility is formed during overexpression of the protein in the cytosol of prokaryotes. In this case, proNGF prepared by recombination remains in the cytoplasm in an insoluble and aggregated form. These protein aggregates, the isolation thereof as well as their purification are described for example in Marston, F. A., Biochem. J. 240 (1986). To isolate these inactive protein aggregates (inclusion bodies) the prokaryotic cells are disrupted following fermentation.

Cell disruption may be performed by conventional methods, e.g. by means of sonication, high pressure dispersion or lysozyme (Rudolph, R., et al. (1997); Folding proteins. In: Creighton, T. E. (ed.): Protein Function: A Practical Approach. Oxford University Press, pp. 57-99). It is preferably carried out in a buffer solution suitable to adjust a neutral to weakly acidic pH value and serving as a suspension medium, such as 0.1 mol/l Tris/HCl. After cell disruption, the insoluble components (inclusion bodies) are removed in any suitable manner, preferably by centrifugation or filtration following one or more washing steps with agents that leave IBs intact but possibly dissolve foreign cellular proteins, e.g. in water or phosphate buffer, optionally with mild detergents added such as Brij®. Afterwards the insoluble fraction (pellet) is subjected to the method according to the present invention for solubilization and naturation.

As the denaturing agent there is conveniently used a denaturing agent usually employed in the solubilization of inclusion body proteins. Guanidinium hydrochloride and other

guanidinium salts, such as the thiocyanate as well as urea and its derivatives are preferably used. Moreover, mixtures of these denaturing agents may be used.

The concentration of the denaturing agent is dependent on the type of denaturing agent and can be determined easily by the skilled artisan. The concentration of the denaturing agent (denaturing concentration) is sufficient if complete solubilization of the denatured protein having a poor solubility may be achieved. For guanidinium hydrochloride, these concentrations usually are in the range of 3 to 8 mol/l, preferably 5 to 7 mol/l. For urea, the concentrations usually are in the range of 6 to 10 mol/l. A weekly denaturing solution is a solution which contains a denaturing agent in a concentration enabling formation of the correct disulfide bonds in the protein and thereby the formation of the native tertiary structure of the protein. Preferably, strongly and weekly denaturing solutions differ in their concentrations by a factor of 100 or more.

Furthermore, for complete monomerization of the inclusion body proteins it is advantageous to also add during the solubilization a reduction agent such as dithiothreitol (DTT), dithioerythritol (DTE) or 2-mercaptoethanol in a concentration of 10-400 mM and particularly preferred in a concentration of 20-100 mM.

Following solubilization a dialysis is performed, preferably against a solution which contains a denaturing agent in a denaturing concentration in order to remove the reduction agent which may optionally be present. Conveniently, the solution against which dialysis is carried out contains the

denaturing agent in the same concentration as present in the denaturing solution.

Subsequent naturation according to the method of the present invention is performed at a pH in the neutral to alkaline range, preferably between pH 7 and 10, particularly preferred in a pH range between 7.5 and 9.5. As the buffer solutions, any conventional buffer may be used. Preferably, buffers known to those skilled in the art such as Tris or phosphate buffers are used as the renaturing agents. To transfer the denatured protein into renaturation buffer, the solubilized protein is either diluted into the renaturation buffer or dialyzed against renaturation buffer. Thereby, the concentration of the denaturing agent is also diluted (weakly denaturing solution) so that no further denaturation of the protein occurs. Already during initial reduction of the concentration of the denaturing agent a renaturing process may occur. The conditions for transfer of the protein into the solution which is not or only weakly denaturing must be properly selected to ensure that the protein substantially remains in solution. Conveniently, this may be achieved by a slow continuous or a stepwise dilution. It is preferred to dilute the denaturing agent in a manner that the naturation of the protein is as complete as possible or the denaturing agent is almost completely removed, e.g. by dialysis.

Preferably, naturation is performed in the presence of low molecular weight auxiliary agents having a positive effect on the yield upon naturation. Such auxiliary agents are for example described in US patent 5,593,865. Particularly preferred as the low molecular weight auxiliary agent during naturation is arginine, conveniently in a concentration of 0.2 to 1.5 M.

According to the method of the present invention, naturation is preferably performed by adding a thiol component in its reduced and oxidized forms. Preferred thiol components include glutathion in the reduced (GSH) and oxidized form (GSSG), cysteamine and cystamine, cysteine and cystine or 2-mercaptoethanol and 2-hydroxy ethyldisulfide. By addition of these thiol reagents in reduced and oxidized forms it is possible to achieve the formation of disulfide bonds within the folding polypeptide chain during renaturation as well as "reshuffling" of wrong disulfide bonds within or between the folding polypeptide chains (Rudolph et al., 1997, loc. cit.).

Conveniently, the method according to the present invention is performed during naturation at low temperatures (preferably at about 10°C). In the course of the method according to the present invention the renaturation is performed for a period of 0.5 to 5 h, preferably 1 to 2 h.

To prevent oxidation of the reducing agent by oxygen present in the air and to protect free SH groups it is convenient to add a complexing agent such as EDTA, preferably in an amount of 1-20 mM, particularly preferred at about 10 mM.

The term "activity of ß-NGF" means the biological activity of ß-NGF. Biologically active ß-NGF exists in the form of a dimer. The activity may be determined according to the DRG assay (dorsal root ganglion assay), Levi-Montalcini, R., et al., Cancer Res. 14 (1954) 49, and Varon, S., et al., Meth. in Neurochemistry 3 (9172) 203. In this assay the stimulation and survival of sensory neurons from dissociated dorsal root ganglia of chick embryos is monitored by means of neurite formation.

The prosequence is a domain separate from the mature protein. Between these two domains there is an exposed protease cleavage site. These cleavage sites may be specifically processed by suitable proteases. For example, trypsin cleaves after basic amino acids such as lysine or arginine. If the ratio of proNGF to trypsin is appropriately adjusted, the correctly folded, mature protein will not be cleaved by this protease. In contrast, denatured proteins as well as folding intermediates expose sequences which are susceptible to an attack by the protease. Proteases having a trypsin-like substrate specificity are preferred for processing of proNGF. These proteases cleave the protein without digesting the active portion of the protein molecule. As the trypsin-like proteases, several serine proteases (e.g. trypsin itself or γ-NGF) are considered. Trypsin is preferably used. For limited proteolysis, the protein is employed in a mass ratio of 1:40 to 1:2500 (trypsin:proNGF ratio), preferably in a range of 1:40 to 1:250. The proteolysis is carried out using an incubation time of 1 min to 24 h, preferably 1 to 60 min at a temperature of 0°C to 37°C, preferably 0°C to 20°C. As the buffers there are used buffers which do not inhibit the activity of the protease. Phosphate and Tris buffer in a concentration range of 10-100 mM are preferred. The limited proteolysis is performed in the optimal pH range of the protease; a medium of pH 7-8 is preferred. After completion of the incubation time the proteolysis is stopped either by addition of a specific inhibitor, preferably 1 to 5 mM PMSF (phenylmethylsulfonylfluoride) or soy bean trypsin inhibitor, preferably 1 mg per 0.1 to 5 mg trypsin, or by reduction of the pH to 2-3 by addition of an acid, preferably HCl (Rudolph, R., et al. (1997); Folding proteins. In: Creighton,

T. E. (ed.): Protein Function: A Practical Approach. Oxford University Press, pp. 57-99; US patent 5,683,894).

The following Examples, publications and Figures further illustrate the present invention the scope of which is obvious from the present Claims. The processes described are meant to be exemplary and describe the object of the present invention also following modification.

Figure 1 shows the proNGF plasmid construct pET11a-proNGF for the expression of recombinant human proNGF.

Figure 2 shows a Coomassie stain of an SDS PAGE gel (15%) of crude extracts of E. coli strain BL21 (DE3) pET11a-proNGF/pUBS520 prior to and after induction, respectively, as well as of an IB preparation (SDS PAGE according to Laemmli, UK, Nature 227 (1970) 680). U: crude extract prior to induction, I: crude extract after four hours induction, P: IB pellet, S: soluble supernatant).

Figure 2a shows the effect of the pH value on the folding of rh proNGF at 10°C in 100 mM Tris/HCl, 1 M L-arginine, 5 mM GSH, 1 mM GSSG, 5 mM EDTA. The protein concentration was 50 µg/ml, the folding period was 3 hours. The mean values of two measuring series are shown.

Figure 2b represents the effect of different concentrations of L-arginine on the folding of rh proNGF. Renaturation took place at a pH of

9.5, the other conditions were identical to those used in pH variation. The mean values of two measuring series are shown.

Figure 2c shows the effect of different GSH concentrations on the folding of rh proNGF. The concentration of GSSG was 1 mM, the L-arginine concentration 1 M. The other parameters of renaturation were identical to those used in arginine variation. The mean values of two measuring series are shown.

Figure 2d shows the effect of different GSSG concentrations on the folding of rh proNGF. The concentration of GSH was 5 mM. The other folding parameters were identical to those used in GSH variation. The mean values of two measuring series are shown.

Figure 2e shows the effect of different amounts of GdmCl on the yield of native rh proNGF. The amount of GSH and GSSG was 5 mM and 0.5 mM, respectively. The other renaturation conditions were identical to those used in GSSG variation. The mean values of two measuring series are shown.

Figure 2f shows the effect of different protein concentrations on the yield of folding of rh proNGF. In all samples, the concentration of GdmCl was 200 mM. All other folding parameters were identical to those used in GdmCl variation. A single measuring series is shown.

Figure 3 shows the elution profile of the purification of rh proNGF by means of cation exchange chromatography on Poros 20 HS (Perseptive Biosystems, column volume 1.7 ml).

Figure 4 shows an SDS PAGE gel (15%, silver stain according to Nesterenko, M. V., et al., J. Biochem. Biophys. Methods 28 (1994) 239) of the purification of rh proNGF on Poros 20 HS (1: renatured proNGF as loaded to the column; 2: void; 3: fraction 4 (66 to 69 ml); 4: fraction 5 (69 to 72 ml); 5: fraction 6 (72 to 75 ml); 6: fraction 7 (75 to 78 ml); 7: fraction 8 (78 to 81 ml); 8: fraction 9 (81 to 84 ml); 9: fraction 10 (84 to 87 ml)).

Figure 5 shows the UV spectrum of rh proNGF.

Figure 6 shows an IEX-HPLC elution diagram of rh proNGF (column material: Poros 20 HS, 100 mm x 4.6 mm column, Perseptive Biosystems company).

Figure 7 shown an RP-HPLC elution diagram of rh proNGF (column material: Poros 10 R1, 100 mm x 4.6 mm column, Perseptive Biosystems company).

Figure 8 shows an SDS gel (15% Coomassie stain) of the limited proteolysis of rh proNGF with trypsin (M: 10 kDA marker, 1: rh proNGF standard; 2: rh ß-NGF standard; 3: mass ratio trypsin:rh proNGF ≈ 1:40, 4: 1:100, 5: 1:250, 6: 1:500,

7: 1:1000, 8: 1:2000, 9: 1:2500, 10: control without trypsin, with STI).

SEQ ID NO: 1 and 2 show oligonucleotides for the construction of pET11a-proNGF.

SEQ ID NO: 3 shows the nucleotide sequence of the cDNA of human proNGF as well as the amino acid sequence of the translation product.

SEQ ID NO: 4 shows the amino acid sequence of the translation product.

Example 1

Cloning of the cDNA encoding proNGF into an E. coli expression vector

For the cloning of the proNGF construct the T7 expression system of Novagen was chosen (Studier, F. U., et al., J. Mol. Biol. 189 (1986) 113). The DNA sequence encoding proNGF is under the control of the strong T7 transcription signal. As the host strain, E. coli BL21 (DE3) is used. The chromosome contains the gene for T7 RNA polymerase. Expression of this RNA polymerase and thereby of the proNGF is induced by IPTG (isopropyl-ß-D-thiogalactoside).

The cDNA for human proNGF was obtained by PCR amplification from vector pMGL-SIG-proNGF of Boehringer Mannheim (PL No. 1905). At the 5' end of the DNA sequence encoding proNGF an NdeI restriction site and at the 3' end a BamHI restriction site were introduced using mutagenesis primers. The PCR product was inserted into the NdeI/BamHI restriction site of

the multiple cloning region of vector pET11a (Novagen) (Fig. 1).

The following primers were used in the PCR:

Forward primer "FwProNGF":

5'-CG GAA TTC CA|T ATG GAA CCA CAC TCA GAG AGC-3' **(SEQ ID NO: 1)**
Met Glu Pro HisSer Glu Ser

Reverse primer "RevNGF":

5'-CC G|GA TCC TTA TCA TCT CAC AGC CTT TCT AGA-3' **(SEQ ID NO: 2)**
stopstopArg Val Ala Lys Arg Ser

After cloning into the vector, the nucleotide sequence was verified by means of DNA sequencing.

Example 2

a) Expression of human proNGF in E. coli

For culturing of the recombinant bacterial strain an overnight culture was prepared. For this purpose a suitable volume of LB medium was added with 100 µg/ml ampicillin and 50 µg/ml kanamycin.

LB medium (1 l): **10 g trypton**
10 g yeast extract
5 g NaCl

The medium was inoculated with a single colony and agitated over night at 37°C.

The next morning, the desired volume of 2xYT medium containing 100 µg/ml ampicillin and 50 µg/ml kanamycin was inoculated with the overnight culture in a ratio of 1:100 (v/v). The culture was agitated at 37°C and 200-250 rpm until OD_{600} of 0.5-0.8 was reached. Afterwards, the expression of proNGF was induced by 3 mM IPTG for 4 h at the same temperature. Subsequently, the cells were harvested by centrifugation and either disrupted immediately or stored frozen at -70°C.

2xYT medium (1 l): **17 g trypton**
10 g yeast extract
5 g NaCl

b) Isolation of IBs

In the bacterial cells the recombinant protein is present in the form of aggregates. The preparation of these "inclusion bodies" was performed according to Rudolph, R., et al. (1987); Folding proteins. In: Creighton, T. E. (ed.): Protein Function: A Practical Approach. Oxford University Press, pp. 57-99.

For cell disruption, 5 g each of the cell pellet were resuspended in 25 ml of 100 mM Tris/HCl pH 7.0; 1 mM EDTA. Afterwards, 1.5 mg of lysozyme were added per g of wet cell mass, incubated for 30 min at 4°C, and subsequently the cells were disrupted using a Gaulin cell disruptor. Then, 3 mM of $MgCl_2$ as well as 10 µg/ml DNase were added to the crude homogenate and incubated for 30 min at 25°C. After DNase digestion the insoluble cell components were solubilized by addition of 0.5 volumes 60 mM EDTA, 6% Triton X-100, 1.5 M

NaCl pH 7.0 followed by incubation for 30 minutes at 4°C. The IBs were collected by centrifugation for 10 min at 13,000 rpm. Afterwards, they were washed four times each with 100 ml of 100 mM Tris/HCl pH 7.0; 20 mM EDTA and stored at -20°C.

In this manner, about 4 g of IB pellet could be reproducibly obtained from 10 l of E. coli culture (about 44 g wet cell weight). The preparations always contained approx. 90-95% rh proNGF (Fig. 2).

Example 3

a) Solubilization of IBs

400 mg of IB pellet were suspended in 2 ml solubilization buffer (100 mM Tris/HCl pH 8.0; 6 mM GdmCl; 100 mM DTT; 10 mM EDTA), incubated for 2 h at 25°C and centrifuged for 30 min at 13,000 rpm in the cold room. Afterwards, the supernatant was removed and adjusted to pH 3-4 with 1 M HCl. The solubilized material was dialyzed three times each against 300 ml 6 M GdmCl pH 4.0, 10 mM EDTA, i.e. twice for 2 h each at 25°C and once over night in the cold room (12°C, 16-18 h). The protein concentration was then determined using the method of Bradford (Bradford, M. M., Anal. Biochem. 72 (1976) 248). The concentration of rh porNGF was between 40 and 50 mg/ml.

b) Optimizing the renaturation of rh proNGF

To prepare biologically active rh proNGF from the solubilized materials prepared in Example 3a) these were diluted into different renaturation buffers. To determine the optimal

folding conditions, the following parameters were varied in the order listed:

a) temperature and time
b) pH
c) arginine concentration
d) GSH/GSSG concentration
e) GdmCl concentration
f) protein concentration

The results are presented in Tables 1 and 2 as well as in Figs. 2a-f. The amount of renatured proNGF in the folding samples was determined by RP-HPLC. For this purpose, 925 µl each of the folding samples were removed at predetermined time points and treated with 75 µl of 32% HCl to stop the folding reaction. For RP-HPLC analytics a Poros 10 R1 HPLC column and the Beckman Gold HPLC system with solvent module 125 NM, detector 168, autosampler 507, and analysis software "Gold V 8.10" were used. The elution peaks obtained were fitted using the "peakfit" program version 2.01. For a quantitative determination of the yields, a standard graph was constructed using purified native rh proNGF. Since the rh proNGF IBs were very pure, the total amount of protein employed in the renaturation samples was equated with the amount of rh proNGF for the quantitative analysis. The measurement results shown are mean values of two measurements each.

Table 1

Determination of the optimal temperature and time during rh proNGF folding. The protein concentration in each of the renaturation samples was 50 µg/ml. The folding buffer consisted of

100 mM Tris/HCl pH 9.5
1 M L-arginine
5 mM GSH
1 mM GSSG
5 mM EDTA

The measurement series were performed several times and fitted using an exponential function. The mean values of two measurements are shown.

Temperature [°C]	Overall yield [%]	No further increase after about	rate constant k [s^{-1}]
4	25.8	3.3 h	2.569×10^{-4} s^{-1}
10	29.0	1.6 h	4.865×10^{-4} s^{-1}
15	22.4	1.1 h	6.399×10^{-4} s^{-1}
20	12.0	1.0 h	1.065×10^{-4} s^{-1}
25	11.4	0.8 h	1.935×10^{-4} s^{-1}

Table 2

This Table shows the effect of different concentrations of GSH/GSSG (GSH = reduced glutathion; GSSG = oxidized glutathion) on the folding of rh proNGF. The renaturation buffer used was

100 mM Tris/HCl pH 9.5
1 M L-arginine
5 mM EDTA

The folding time was 3 h at 10°C. In the Table, the individual folding samples are presented in the order of

decreasing yield. The mean yields of two measurement series are shown.

No. of sample	ratio GSH/GSSG [mM]	yield [%]
1	5/0.5	37.7
2	5/1	35.0
3	5/5	34.0
4	5/2.5	33.1
5	1/1	29.4
6	5/10	27.6
7	5/20	26.0
8	2.5/1	22.1
9	10/1	21.2
10	1/5	18.9
11	20/1	10.9
12	0/1	9.85
13	0/0	0
14	5/0	0

c) Renaturation of rh proNGf in the preparative scale

Rh proNGF was renatured by dilution in folding buffer (100 mM Tris/HCl pH 9.5; 1 M L-arginine; 5 mM GSH; 0.5 mM GSSG; 5 mM EDTA). The folding was preformed at a protein concentration of 50 µg/ml. The renaturation sample was incubated for 3 h at 10°C.

d) Purification by means of ion exchange chromatography

The renatured material was dialyzed against 10 l of 50 mM Na-phosphate pH 7.0; 1 mM EDTA (IEX buffer A) and centrifuged for 30 min at 20,000 rpm. The supernatant was loaded onto a Poros 20 HS column and eluted using a salt gradient (IEX buffer B: 50 mM Na-phosphate pH 7.0, 1 M NaCl, 1 mM EDTA). The protein eluted at 980 mM NaCl (Fig. 3). Non-native rh proNGF can only be removed from the column using denaturing conditions.

Example 4

Characterization of rh proNGF

a) Determining the concentration and the molecular weight by means of UV spectrophotometry

To determine the concentration of rh proNGF in the purified samples, an UV spectrum from 240 to 340 nm was taken of the samples dialyzed against 50 mM Na-phosphate pH 7.0, 1 mM EDTA (Fig. 5; the spectrum was obtained using a Beckman DU 640 spectrophotometer). The rh proNGF concentration in the sample was determined by means of absorption at 280 nm. The evaluation was based on a theoretical molar extinction coefficient of 25,680 l/(mol x cm) (calculated according to Gill, S. C., et al., Anal. Biochem. 182 (1989) 319) and a molecular weight of 24,869 Da per monomer (calculated by means of the ExPASy program "pI/Mw" and corrected for three disulfide bonds). The values obtained using the spectrum were in close correlation to the concentrations determined by means of the Bradford method. Molecular weight determination was done using electron spray mass spectrometry. The

theoretical mass of recombinant proNGF is 24,689 Da. Experimentally determined were 24,871 Da.

b) Analysis of the purity and determination of the molecular weight using SDS polyacrylamide gel electrophoresis

15% polyacrylamide gels were used. Each sample contained 1% (v/v) 2-mercaptoethanol. In the SDS gel, the recombinant human proNGF shows a slightly higher apparent molecular weight than expected: approx. 30 kDa (instead of 24.8 kDa) (Fig. 2).

c) Analysis of the purity by means of IEX-HPLC

24 µg (50 µl of a sample containing 0.48 mg/ml rh proNGF) of protein were loaded onto a Poros 20 HS column (125 x 4 mm) equilibrated with 50 mM Na-phosphate pH 7.0; 1 mM EDTA, and were eluted at a flow rate of 5 ml/min with a linear gradient of 0 to 100% B (B = 50 mM Na-phosphate pH 7.0; 2 M NaCl; 1 mM EDTA) in a period of 10 minutes (Fig. 6). The absorption at 280 nm was used for detection (Gyncotek HPLC system with analysis software Chromeleon version 3.14).

d) Analysis of the purity using RP C4 HPLC

3.1 µg of rh proMGF (15 µl rh proNGF in a concentration of 0.21 mg/ml) were loaded onto a Poros 10 R1 column (100 mm x 4 mm; Perseptive Biosystems) equilibrated with 0.13% TFA. The protein was eluted at a flow rate of 0.8 ml/min with a non-linear gradient (0-4 min: 6% B; 4-9 min: 6-30% B; 9-24 min: 30-69% B; 24-25 min: 69-100% B; 25-30 min: 100% B)) in a period of 33 minutes. As the eluent B there was used 0.1% (v/v) TFA in 80% (v/v) acetonitrile. The absorption at 220 nm

was used for detection (Beckman "Gold" HPLC system with analysis software "Gold V 8.10"). Native rh proNGF eluted in a single peak at a retention time of 14.28 min (Fig. 7).

e) Analysis of the N terminal sequence

For N terminal sequence analysis the solubilized IBs were used which had been roughly purified by means of RP HPLC. The N terminal sequence was determined using an Applied Biosystems 476A protein sequencing device. The following amino acid sequence was obtained:

H_2N-Met-Glu-Pro-His-Ser-Glu-Ser-Asn-Val

f) Biological activity of the recombinant human proNGF

The physiological activity of rh proNGF was determined using the DRG assay (= dorsal root ganglion assay) (Levi-Montalcini, R., et al., Cancer Res. 14 (1954) 49; Varon, S., et al., Meth. in Neurochemistry 3 (9172) 203). In this assay the stimulation and survival of sensory neurons from dissociated dorsal root ganglia of 7-8 day old chick embryos is determined by means of neurite formation. The rh proNGF sample was adjusted to concentrations of 0.019 to 20.00 ng/ml using culture medium. Per test sample 15,000 neurons were employed. After incubation for 48 hours at 37°C the number of surviving cells was determined. A solution of rh ß-NGF of known concentration was used as the reference sample. The quantitative evaluation is based on the so-called EC_{50} value, i.e. the concentration of NGF promoting the survival of half of the neurons. For rh proNGF an EC_{50} value of 0.369 ng/ml was obtained. In comparison, the EC_{50} value obtained for the rh ß-NGF standard was 0.106 ng/ml. Considering the different

molecular weight of rh ß-NGF and rh proNGF, the biological activity of mature rh ß-NGF is about twice as high as that of rh proNGF.

Example 5

a) Preparation of biologically active mature rh ß-NGF by limited proteolysis of rh proNGF

Human proNGF contains an arginine residue as the last amino acid of the prosequence. Therefore, from this precursor the mature rh ß-NGF may be obtained in vitro by limited proteolysis using proteases of suitable substrate specificity such as trypsin.

500 µl of purified rh proNGF were dialyzed against 50 mM Tris/HCl pH 8.0. Following dialysis, a protein concentration of 0.49 mg/ml was measured by running the UV spectrum. Per digestion sample, 20 µg of proNGF were employed. After proteolysis, 3 µg (corresponding to 6 µl) of this sample were analyzed by means of SDS PAGE. As the trypsin stock solution 0.1 µg/ml or 0.01 µg/ml, respectively, were used. The concentration of soy bean trypsin inhibitor (STI) was 1 mg/ml. Both proteins were provided in the form of lyophilized powders (manufacturer: Boehringer Mannheim and Sigma, respectively) and were dissolved in the above-mentioned buffer.

Different mass ratios of trypsin/rh proNGF were used in the limited proteolysis (see Table 3). After an incubation for thirty minutes on ice each reaction was stopped by 5 µg STI. For control purposes rh proNGF without added protease was also incubated on ice, followed by addition of STI.

Table 3

Ratio trypsin:rh proNGF	M (trypsin) [µg]	V (trypsin) [µl]	V (rh proNGF) [µl]	V (STI) [µl]
1:40	0.5	5 (0.1 µg/ml)	40	5
1:100	0.2	2 (0.1 µg/ml)	40	5
1:250	0.08	0.8 (0.1 µg/ml)	40	5
1:500	0.04	4 (0.01 µg/ml)	40	5
1:1000	0.02	2 (0.01 µg/ml)	40	5
1:2000	0.01	1 (0.01 µg/ml)	40	5
1:2500	0.008	0.8 (0.01 µg/ml)	40	5
Control	-	-	20	2.5

g) Analysis of the cleavage products by N terminal sequencing

The digestion samples with a mass ratio of trypsin:rh proNGF of a) 1:40; b) 1:100, and c) 1:250 were subjected to a more detailed analysis by N terminal sequencing. A band at 13 kDa contained several species (Figure 8):

N terminus 1: Met^{-104}....;
N terminus 2: Val^{-35}....;
N terminus 3: Ser^{1}....(mature rh ß-NGF);
N terminus 4: Gly^{10}....;

These peptides were present in the different samples in differing amounts.

Sample a): N terminus 2:N terminus 3:N terminus 4 = 4:5:2.
Sample b): N terminus 2:N terminus 3 = 1:1; N terminus 4 in trace amounts.

Sample c) was analyzed in addition by means of RP C3 HPLC (column: Nucleosil 500-5 C3-PPN; 125 mm x 4 mm). Two peaks were obtained: peak 1 (12.32 min): N terminus 1; peak 2 (14.88 min): N terminus 2 and N terminus 3 in a ratio of 2:3.

To obtain mature rh ß-NGF from rh proNGF on a preparative scale 1.3 mg of rh proNGF (in 50 mM Tris/HCl pH 8.0; concentration 0.46 mg/ml) were added with trypsin in a mass ratio of 1:250 (trypsin:rh proNGF). The sample was incubated for 30 min on ice. Afterwards, the protease was inactivated by a 40fold excess based on the mass of soy bean trypsin inhibitor. The cleavage sample was dialyzed against 50 mM sodium phosphate pH 7.0, 1 mM EDTA and then applied to a cation exchange column (1.7 ml Poros 20 HS; Perseptive Biosystems). In a linear salt gradient of 0 to 2 M NaCl the cleavage product eluted in a single peak. The elution at a salt concentration of about 840 mM NaCl corresponded to that of mature rh ß-NGF in a control experiment. The yield of purified cleavage product was 17%.

The biological activity of the purified cleavage product was tested by means of the DRG assay. It corresponded to the activity of mature rh ß-NGF (Table 4).

Table 4

Species	EC_{50} value [pg/ml]
rh ß-NGF	110
rh ß-NGF prepared by limited proteolysis of rh proNGF	171

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PCT/EP99/07613
Applicant: RUDOLPH, Rainer et al.

Claims

1. A method for the preparation of biologically active ß-NGF from its inactive pro form having a poor solubility which is obtainable after recombinant preparation in prokaryotes, wherein proNGF in its inactive form having poor solubility is solubilized in a solution of a denaturing agent in a denaturing concentration and is afterwards transferred into a solution which is not or weakly denaturing whereby solubility is maintained and the denatured proNGF assumes a biologically active conformation as determined by the disulfide bonds present in native ß-NGF, and subsequently the prosequence is cleaved off whereby active ß-NGF is obtained which can be isolated.

2. A method according to claim 1 wherein the not or weakly denaturing solution contains arginine.

3. A method according to claim 2 wherein the concentration of arginine is 0.2 to 1.5 mol/l.

4. A method according to any of the claims 1 to 3 wherein the naturation is carried out in the presence of a thiol component in its reduced and oxidized form.

5. A method according to any of the claims 1 to 4 wherein the cleaving off of the prosequence is carried out by means of a protease with a substrate specificity for cleaving after the amino acid arginine.

6. A method according to claim 5 wherein trypsin is used as the protease.

7. A method according to any of the claims 1 to 6 wherein guanidinium hydrochloride or urea is used as the denaturing agent.

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Applicant: RUDOLPH, Rainer et al.

Novel Claims 8 and 9

8. A pharmaceutical preparation containing recombinant proNGF as the active ingredient.

9. The use of recombinant proNGF for the preparation of a pharmaceutical preparation for the treatment of neuropathies.

Fig. 1

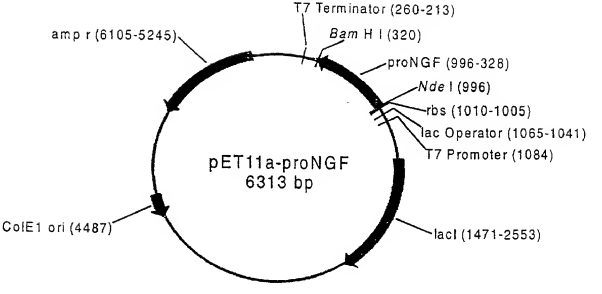


Fig. 2

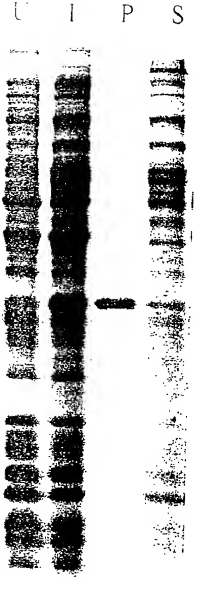


Fig. 2a

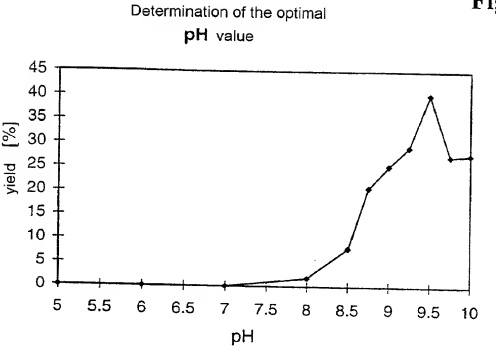

Determination of the optimal
pH value
yield [%]
45
40
35
30
25
20
15
10
5
0
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
pH

Fig. 2b

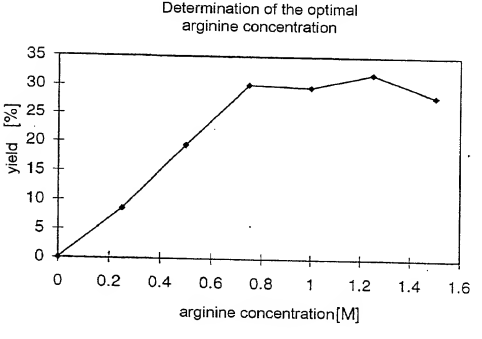

Determination of the optimal
arginine concentration
yield [%]
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
arginine concentration[M]

Fig. 2c

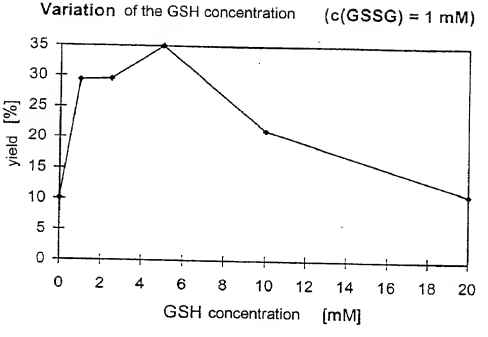

Variation of the GSH concentration (c(GSSG) = 1 mM)
yield [%]
35
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20
GSH concentration [mM]

Fig. 2d

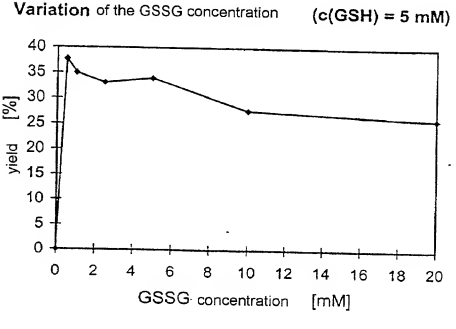

Variation of the GSSG concentration (c(GSH) = 5 mM)
yield [%]
40
35
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20
GSSG concentration [mM]

Fig. 2e

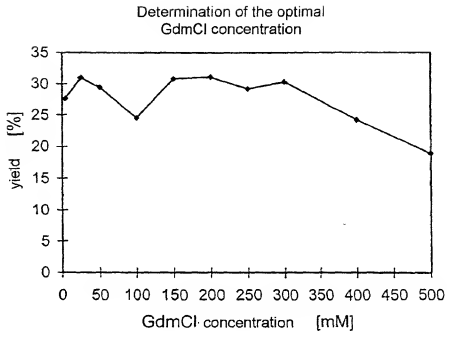

Determination of the optimal
GdmCl concentration
yield [%]
0
5
10
15
20
25
30
35
0 50 100 150 200 250 300 350 400 450 500
GdmCl concentration [mM]

Fig. 2f

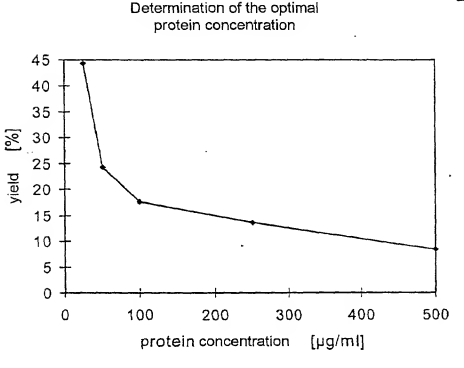

Determination of the optimal
protein concentration
yield [%]
0
5
10
15
20
25
30
35
40
45
0 100 200 300 400 500
protein concentration [µg/ml]

Fig. 3

Elution profile of the purification of rh-proNGF on Poros 20 HS

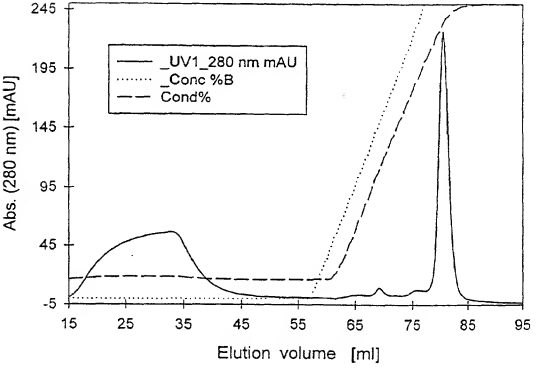


Fig. 4

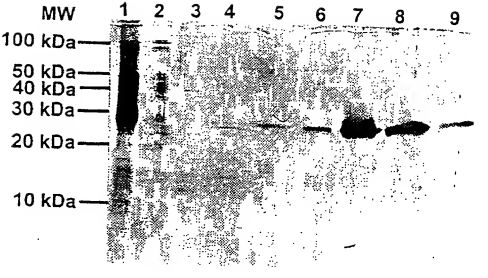


Fig. 5

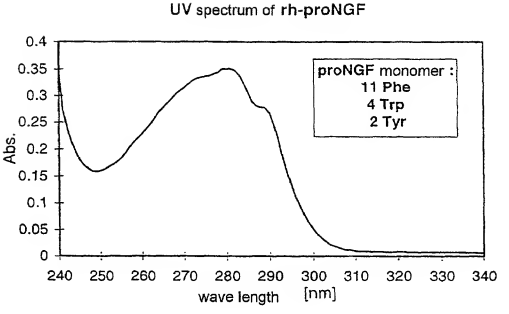


Fig. 6

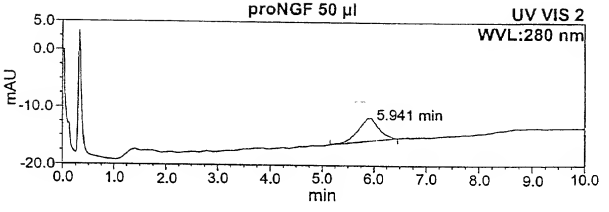


Fig. 7

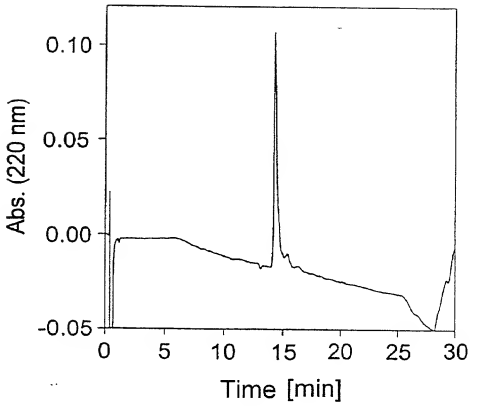
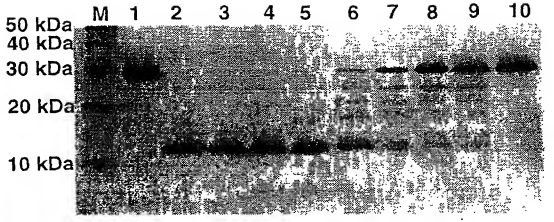


Fig. 8



Attorney's Docket No.: 13028-002001
Client's Ref. No.: P12999

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD FOR OBTAINING ACTIVE BETA-NGF, the specification of which was filed on April 9, 2001 as Application Serial No. 09/807,096and was amended on April 9, 2001.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application No.	Filing Date	Priority Claimed
PCT	PCT/EP99/07613	October 11, 1991	[X] Yes [] No
EP	98119077.0	October 9, 1998	[X] Yes [] No

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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Combined Declaration and Power of Attorney
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